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Applicant(s): John Roderick Morrison, et al.

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For: LONG-TERM CELL-CULTURE COMPOSITIONS AND GENETICALLY MODIFIED ANIMALS DERIVED THEREFROM

Art Unit: Unassigned

Dated: April 10, 2001

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

CLAIM OF PRIORITY

Applicants in the above-identified application hereby claim the right of priority in connection with Title 35 U.S.C. §119 and in support thereof, herewith submit certified copies of:

Australian Application Number PQ 4495 filed December 7, 1999;

Australian Application Number PQ 9242 filed August 7, 2000;

Australian Application Number PR 1109 filed October 31, 2000; and

Australian Application Number PR 1108 filed October 31, 2000.

Respectfully submitted,

Leopold Presser
Registration No. 19,827

Scully, Scott, Murphy & Presser
400 Garden City Plaza
Garden City, NY 11530
(516) 742-4343

LP:JAM:lf

CERTIFICATE OF MAILING UNDER 37 C.F.R. §1.8(a)

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Assistant Commissioner of Patents and Trademarks, Washington, D.C. 20231 on April 10, 2001.

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Mishelle Mustafa

Patent Office
Canberra

I, GAYE TURNER, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PR 1109 for a patent by MONASH UNIVERSITY filed on 31 October 2000.

WITNESS my hand this
Sixth day of March 2001

GAYE TURNER
TEAM LEADER EXAMINATION
SUPPORT AND SALES

AUSTRALIA
Patents Act 1990

PROVISIONAL SPECIFICATION

Invention Title: A NOVEL CELL TYPE FOR NUCLEAR TRANSFER

Applicant: MONASH UNIVERSITY

The invention is described in the following statement:

A NOVEL CELL TYPE FOR NUCLEAR TRANSFER

The present invention generally relates to cells expressing the telomerase catalytic component (TERT cells) and their use in nuclear transfer. The present invention also relates to the use of TERT cells for gene targeting and gene knockout experiments.

INTRODUCTION

The successful development of normal animals from a number of mammalian species using somatic cell nuclear transfer techniques has lead to the possibility that this approach may be used for the production of large numbers of genetically modified livestock and animals for biomedical research. However, one of the major limitations to this technology is found in the normal life span of the somatic cells generally used as the source of donor nuclei in the nuclear transfer procedures. Mammalian somatic cells have a limited life span and enter senescence after a limited number of cell divisions. Because the successful integration or deletion of a DNA sequence in cells in culture requires a relatively large number of cellular divisions, this limit on cell proliferation represents an obstacle to the genetic manipulation of the donor cell nuclei and, ultimately, to the production of genetically modified animals by nuclear transfer. The production of somatic cells capable of continuous growth in culture and their application to nuclear transfer would represent a major step towards the production of such genetically modified animals.

The discussion of documents, acts, materials, devices, articles and the like is included in this description solely for the purpose of providing a context for the present invention. It is not suggested or represented that any or all of these matters formed part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia.

Accordingly, it is an object of the present invention to overcome or at least alleviate some of the problems with the prior art.

SUMMARY OF THE INVENTION

In a first aspect of the present invention there is provided a method of producing an animal, said method comprising introducing a continuously growing donor cell nucleus from a continuously growing donor cell into an oocyte or embryo and allowing the resulting embryo to mature and to preferably develop to a foetus or an adult animal.

In a preferred aspect of the present invention, the donor cell is a genetically modified somatic cell. Preferably, the donor cell is derived from a non-transformed immortalised cell line that expresses telomerase catalytic component (TERT), which allows the cell to grow continuously in culture thereby enabling repeated genetic manipulations of the cell. Similarly, the nucleus may be derived from the immortalized cell line or genetically modified somatic cell which is continuously growing.

In another preferred aspect of the present invention, the donor cell is a further genetically modified TERT cell, said TERT cell comprising a foreign gene which has been introduced into the TERT cell.

In another preferred aspect, the nucleus is derived from a genetically modified TERT cell comprising a foreign gene which has been introduced into the TERT cell.

In yet another preferred aspect of the present invention, the donor cell is a further genetically modified TERT cell, said TERT cell having a destroyed, modified or deleted gene. Such genetically modified TERT cells are useful in gene targeting and gene knockout experiments.

In yet another preferred aspect, the nucleus is derived from a further genetically modified TERT cell, said TERT cell having a destroyed, modified or deleted gene.

In another aspect of the present invention there is provided a method of producing a cell line that may be expanded from an embryo to produce cloned cells of an embryo, said method comprising

introducing a continuously growing donor cell or nucleus from a continuously growing cell, into an oocyte or embryo;

culturing the oocyte or embryo to an advanced cleavage stage embryo;

separating and cloning the cleaved cells of the embryo; and

optionally culturing the cloned cells.

In another aspect of the present invention there is provided an animal produced by the methods of the present invention. Preferably, the animal is a genetically modified animal, preferably the genetically modified animal is a knockout animal.

Throughout the description of the present invention the use of the terms "TERT cell" or "TERT cells" is used to indicate any cell or cells which have been modified to express the telomerase catalytic component to allow them to grow continuously in culture.

DESCRIPTION OF THE INVENTION

In a first aspect of the present invention there is provided a method of producing an animal, said method comprising introducing a continuously growing donor cell nucleus from a continuously growing donor cell into an oocyte or embryo and allowing the resulting embryo to mature and to preferably develop to a foetus or animal.

It is desirable to use a donor cell or cells which have the ability to grow continuously in culture. Some cells have the limitation of being short lived and they stop dividing in a very short period. Accordingly there is little time for genetic manipulation of these cells and this is often a major limitation in genetic modification or knockout studies. Some cell lines which are naturally continuously growing (ie neuronal stem cells) and do not require further genetic

manipulation may also be used. From these cells, the nucleus may also be extracted and used in the present invention.

In a preferred aspect of the present invention, the donor cell is a genetically modified somatic cell. Similarly, the nucleus may be derived from a genetically modified somatic cell which is continuously growing.

Preferably the donor cell nucleus is derived from a non-transformed cell line. Manipulation or genetic modification of the cell line by any method that immortalizes the cell line may be used.

The following description exemplifies a type of cell line which is capable of continuous growth. However, it should be appreciated that the invention should not be restricted to this cell line or the nuclei derived from these cells as the invention is applicable to all cell lines capable of continuous growth and immortality. The following description is merely illustrative and should not be taken as a restriction on the generality of the invention.

The expression of telomerase catalytic component (TERT) in a cell may induce the cell to immortalize and undergo continuous growth in culture. Accordingly, it is preferred that the cells are manipulated to express telomerase catalytic component (TERT). However, cells already expressing TERT and which are not genetically modified may be used in the present invention. More preferably, the gene encoding TERT is introduced into the cell. This can result in a cell line that is immortalized. The expression of TERT in the cells may also allow the cells to undergo (repeated) genetic manipulations as the cells can be grown continuously in culture for many weeks and/or months. TERT may be inserted into the cell line of choice using standard transfection technologies. The cell or cells may be from any cell type, preferably the cell or cells are fibroblast somatic cells.

The term "TERT cell(s)" as used herein means a cell which expresses TERT either naturally or by introduction via genetic manipulation.

TERT may be cloned from cells expressing this gene (eg embryonic tissue may be used) alternately the cDNA for TERT is commercially available.

The TERT cells may also be obtained from a genetically modified animal which has been manipulated to express TERT in it's somatic cell lineages. TERT cells may be collected from any stage of development of the animal. Preferably the source of TERT cells is from a foetus which is differentiated at a stage after the embryonic stage. The whole foetus or a part thereof may be used as a source of the TERT cells. Preferably the cells are obtained from a rat expressing TERT in its somatic cell lineages.

Oocytes may be obtained from any source. For example, they may be of bovine, ovine, porcine, murine, caprine, amphibian, equine or of a wild animal origin. Preferably the oocyte is a rodent oocyte. More preferably it is a rat oocyte.

The entire contents of PCT/AU97/00868 are hereby incorporated and referred to in this description particularly with respect to the oocytes suitable for this invention and of the enucleation of suitable oocytes.

The TERT cell or cells may be introduced into the oocyte or embryo using any method available to the skilled addressee. Preferably nuclear transfer procedures are used. More preferably a TERT cell is injected into an enucleated oocyte, the oocyte is activated to initiate development and the resulting embryo is transferred to a receptive recipient animal capable of supporting the development of the embryo into a foetus or animal. Other methods may be used to introduce the cell into an oocyte or embryo including but not limited to aggregation of the TERT cell or cells with preimplantation embryos or injection of the TERT cell or cells into the cavity of a blastocyst stage embryo.

The entire contents of PCT/AU99/00275 are hereby incorporated and referred to in this application, particularly for the description of nuclear transfer of donor cells into oocytes.

In a preferred aspect of the present invention, the donor cell is a further genetically modified TERT cell, said TERT cell comprising a foreign gene which has been introduced into the TERT cell.

In another preferred aspect, the nucleus is derived from a genetically modified TERT cell comprising a foreign gene which has been introduced into the TERT cell.

A genetically modified TERT cell refers to a TERT cell into which a foreign (ie non-naturally occurring) nucleic acid, eg, DNA, has been introduced. The foreign nucleic acid may be introduced by a variety of techniques, including, but not limited to, calcium-phosphate-mediated transfection, DEAE-mediated transfection, microinjection, retroviral transformation, electroporation, immunoporation, protoplast fusion and lipofection. The genetically modified cell may express the foreign nucleic acid in either a transient or long-term manner. In general, transient expression occurs when foreign DNA does not stably integrate into the chromosomal DNA of the transfected cell. In contrast, long-term expression of foreign DNA occurs when the foreign DNA has been stably integrated into the chromosomal DNA of the transfected cell.

Foreign (heterologous) nucleic acid may be introduced or transfected into TERT cells. A TERT cell which harbours foreign DNA is said to be a genetically modified TERT cell. The foreign DNA may be introduced using a variety of techniques known to the skilled addressee. In a preferred embodiment, foreign DNA is introduced into TERT cells using the technique of retroviral transfection. Recombinant retroviruses harbouring the gene(s) of interest are used to introduce into TERT cells using the technique of retroviral transfection. Recombinant retroviruses harbouring the gene(s) of interest are used to introduce marker genes, such as but not limited to βgalactosidase (lacZ) gene, or oncogens. The recombinant retroviruses are produced in packaging cell lines to produce culture supernatants having a high titer of virus particles (generally 10.sup.5 to 10.sup.6 pfu/ml). The recombinant viral particles are used to infect cultures of the TERT cells or their progeny by incubating the cell cultures with medium containing the viral particles and 8μg/ml polybrene for

three hours. Following retroviral infection, the cells may be rinsed and cultured in standard medium. The infected cells may be then analysed for the uptake and expression of the foreign DNA. The cells may be subjected to selective conditions which select for cells that have taken up and expressed a selectable marker gene.

In a preferred aspect of the present invention, the donor cell is a further genetically modified TERT cell, said TERT cell having a destroyed, modified or deleted gene. Such genetically modified TERT cells are useful in gene targeting and gene knockout experiments.

These genetically modified TERT cells include the above genetically modified TERT cell wherein the introduced foreign gene is modified or mutated after genetic modification.

In yet another preferred aspect, the nucleus is derived from a further genetically modified TERT cell, said TERT cell having a destroyed, modified or deleted gene.

In another aspect of the invention, there is provided an embryo, and wherein said embryo results from introducing a continuously growing donor cell nucleus from a continuously growing donor cell into an oocyte or embryo as prepared by the method as described herein. The embryo is preferably a transplantation embryo.

In another aspect of the present invention there is provided a method of producing a cell line that may be expanded from an embryo to produce cloned cells of an embryo, said method comprising

introducing a continuously growing donor cell nucleus from a continuously growing donor cell into an oocyte or embryo;

culturing the oocyte or embryo to an advanced cleavage stage embryo;

separating and cloning the cleaved cells of the embryo; and

optionally culturing the cloned cells.

Once the cell lines are cloned, these may be used to generate genetically identical lines and animals. This technique may be particularly useful for non-murine models such as monkeys to develop genetically identical animals.

The cells of such a nuclear transplantation embryo may be recycled to provide donor cells for further cycles of nuclear transfer, as described in Australian patent 687422 to the present applicant, the entire disclosure of which is incorporated herein by reference.

Accordingly, in another aspect, the present invention provides a cell line expanded from an embryo as prepared by the methods described herein.

In a further aspect of the present invention there is provided an animal produced by the methods of the present invention. Preferably, the animal is a genetically modified animal, preferably the genetically modified animal is a knockout animal.

The transplantation embryos produced by the methods of the present invention may be used to produce genetically identical or similar animals by transplantation into a recipient female, preferably a synchronised female. Preferably, the recipient female is synchronised using fertility drugs, steroids or prostaglandins. Methods for transfer of embryos to recipient females are known to those skilled in the art.

A genetically modified animal may include the addition of foreign genes capable of identification by the presence of marker genes which have been introduced into a donor cell or nucleus. Suitable marker genes may include fluorescently labelled genes which may facilitate identification of genetically modified animals. A genetically modified animal may include a transgenic animal.

Genetically modified animals may also include knockout animals having genes targeted, destroyed and/or modified so that an animal is developed without the gene. Genes may be modified by removal from the genome or by point or random mutations in a gene.

Accordingly, the present invention preferably provides knockout animals which may be useful for research in gene function, diseases, drug therapies and gene development of animal strains having knockout genes.

The genetically modified animals may be useful for research purposes at any stage of development, preferably adult knockout animals are obtained however animals at any stage of development may be used.

Preferably the animal is a mammal including but not limited to murine, bovine, ovine, porcine, equine, feline, endangered species, live stock or may derive from marsupials including kangaroos, wombats. Preferably the animal is murine. Most preferably the animal is a rat.

Throughout the description of the present invention the use of the term "TERT cell" or "TERT cells" is used to indicate any cell or cells which have been modified to express the telomerase catalytic component to allow them to grow continuously in culture.

Throughout the description and claims of the specification, the word "comprise" and variations of the word, such as "comprising" and "comprises", is not intended to exclude other additives, components, integers or steps.

The present invention will now be more fully described with reference to the following examples. It should be understood, however, that the description following is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

EXAMPLES

Example 1 : Media for growing rat foetal fibroblasts

F12 nutrient media (Gibco BRL, Life Technologies) containing 10,000 U of penicillin and 500 U streptomycin, 15% foetal calf serum (ES cell grade, Gibco BRL) was used for the culture and propagation of foetal fibroblasts. This basis media is designated F12/FCS media.

Example 2 - Preparation of fibroblasts cells

A pregnant rat (eg. Sprague-Dawley) was humanely killed at 10.5-16.5 days gestation by CO_2 asphyxiation. Foetuses were removed and placed into a tube with PBS containing penicillin/streptomycin.

Membranes from the foetuses were removed and their heads were separated from their bodies. The pooled carcasses were placed into a small dish (6cm) and the tissue was minced with a blunt object (the tip of a syringe) until it was homogeneous in size. A syringe was used to aspirate the minced tissue which was then transferred into a tube. The dish was washed with 5-10 ml PBS and then aspirated into the syringe and pooled into the tube containing the tissue.

The minced tissue was left to settle at the bottom of the tube for a few minutes and was carefully aspirated off the liquid. The tissue was washed with fresh PBS until it was reasonably clear (approximately 2 washes). 5 ml of Trypsin 0.1% in versene), was added to the tissue and the tube was placed into a 37°C water bath, at 37°C for no longer than 15 min (The tubes were mixed occasionally). The tissue was allowed to settle down to the bottom of the tube and the cell suspension was transferred into a centrifuge tube. The tissue was washed in 5ml F12 media containing FCS, and the cell suspension was pooled with the trypsin cell suspension. Cells are then plated on a standard tissue culture flask and allowed to proliferate. Cells are propagated in F12 media containing FCS according to standard procedures.

Example 3: Preparation of TERT fibroblasts

A mammalian expression vector expressing TERT may be obtained using standard cloning procedures, familiar to anyone experienced in the art, Alternately the TERT expression vector is commercially available.

For stable transfection experiments vectors are linearised at unique restriction endonuclease site. Transfection experiments were initiated on Day 3 of culture in 10 cm dishes using Lipofectamine® Plus. Transfection involved addition of 8μg of linearised plasmid to 20μl of Plus® reagent in 750 μl of serum-free (SF) media with incubation at 23°C for 15 minutes. 30μl of Lipofectamine® was then added to 720μl of F12/FCS media and the solutions were then mixed together and incubated at 23°C for a further 15 minutes. Media was then aspirated from the cells and replaced with 5ml of SF media. The DNA/Lipofectamine® solution was then added to the cells followed by the addition of 6.5ml of F12/FCS 2-3 hours later. On the following day media was replaced with F12/FCS media containing a selectable marker (that was included in the original TERT construct) For example in our experience 300μg/ml of Geneticin® (Gibco BRL Life Technologies) or 50μg/ml of hygromycin are suitable concentrations for the rat foetal fibroblasts . Antibiotic selection was continued for a period of 10 days (ie. Day 14). Following this initial selection processes the cells are maintained on 0.5 X the original concentration of antibiotic.

Example 4: Nuclear Transfer using fibroblast cells as donor nuclei

Animals were killed by decapitation and the oviducts removed in less than 5 minutes. Oviducts were collected into prewarmed calcium free phosphate buffered saline (PBS). Oocytes were liberated from the oviducts into M16 culture medium containing 40 IU/ml hyaluronidase at 37 °C using fine forceps. Oocytes were washed twice in M2 medium after 5 minutes exposure to hyaluronidase. Cumulus free oocytes were transferred to equilibrated modified rat embryo culture medium (MR1ECM) and incubated in humidified 5 % CO_2 in air at 37 °C until use.

Oocytes at the metaphase II stage (i.e. with the first polar body extruded) were selected for nuclear transfer (NT).

Oocytes were enucleated in handling media containing cytochalasin B (7.5µg/ml, Sigma) by gentle aspiration of the polar body and metaphase plate in a small amount of cytoplasm using a glass pipette (inner diameter: 10-15µm).

After mechanical disruption of the donor cell membranes in Hepes buffered TCM199 with 5% rat serum (199HF) using the injection pipette, the fibroblast nuclei were injected directly into the oocyte cytoplasts. The reconstructed embryos were transferred back into MR1ECM until activation.

Artificial activation was induced 4 hours after injection by exposing the oocytes to 8% ethanol in phosphate buffered saline for 5 minutes, prior to culture in MR1ECM containing 35µM cychloheximide for five hours.

Embryos were cultured in modified MR1ECM culture media (Oh et al, (1998) Biol Reprod.59:884-889) supplemented with 10% Rat Serum in a 5% CO_2 Incubator at 37°C.

Embryos were transferred back to primed recipient animals on day 2, 3 or day 4 of culture.

The above example is also applicable for the TERT fibroblasts prepared as in Example 3.

Finally, it is to be understood that various other modifications and/or alterations may be made without departing from the spirit of the present invention as outlined herein.

DATED: 31 October 2000
PHILLIPS ORMONDE & FITZPATRICK
Attorneys for:
MONASH UNIVERSITY

David B Fitzpatrick